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PRINCIPAL INVESTIGATOR: Larry J. Moore, Ph.D.
Muralidhara Padigaru, Ph.D.

CONTRACTING ORGANIZATION: Eastern Analytical, Incorporated

Gaithersburg, MD 20877

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Larry J. Moore, Ph.D
Muralidhara Padigaru, Ph.D

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The availability of potable water in the field is essential to protect the health of soldiers in combat and training. Increasingly, the danger of coliform bacteria and other pathogens in water supplies has become a major concern. Conventional methods based on cell culture techniques for the detection of pathogens or their indicator organisms can require 24 hours or more to complete. Moreover, the detection and measurememt techniques used are often complicated and not portable. Recent and on-going advances in gene amplification and related measurement technologies have permitted in this Phase II SBIR project the development of a potentially portable process to determine *E. coli* bacteria in water in less than three hours and with a sensitivity of one bacterium. The individual components employed in the water characterization process can easily be combined into a portable packge for field evaluation of water quality. Subsequent improvements in the technology are expected to permit a near on line measurement capability and to extend the capability to other pathogens in an easy-to-use portable field kit configuration.

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FOREWORD

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In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 32 CFR 219 and 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

Principal Investigator's Signature

October 10, 1996
Date

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INTRODUCTION

Increasingly stringent requirements are being mandated for the monitoring of pathogens in water supplies. The U.S. Environmental Protection Agency (EPA) has promulgated a series of monitoring and characterization requirements for coliform bacteria and other potential contaminants, as specified in the National Primary Drinking Water Regulations (1). These requirements have been strengthened through recent legislation and promulgation by the EPA in May of this year that stipulate monitoring requirements for public drinking water supplies, including cryptosporidium, giardia, viruses, disinfection byproducts, water treatment plant data and other information requirements (2). This recent EPA ruling for information collection establishes monitoring and data reporting requirements for large public water systems, especially (a) for disinfection byproducts, and (b) for disease-causing microorganisms (pathogens), including cryptosporidium.

These, and previous (3), drinking water monitoring requirements promulgated by the EPA serve as the basis for the analytical measurements approved for use in monitoring the coliform bacteria and other contaminants, and also serve currently as the basis for water monitoring procedures employed by the military to assess potable water supplies. The methods currently approved by the EPA and used by the military for coliform bacteria utilize a time-consuming culturing technique to monitor the potability of water used in the field for military operations. Water used by the military in the field is usually purified by reverse osmosis technology, using large scale mobile facilities that can be used to tap into the local water supplies during field operations. Typically the water is monitored for contamination, using current EPA procedures, at

water sampling, transportation, and consumption sites, since the water is usually consumed at a site remote from its initial production.

The availability of potable water is essential to protect the health of soldiers in combat and training. Current methods for the detection of pathogens or their indicator organisms typically require at least 24 hours, and perhaps as much as several days (4). Recent and ongoing advances in gene amplification and related technologies offer the promise of developing a system that can be used to characterize these pathogens or their indicators in a fraction of the time now required (5-8). By building upon this previous research and by the addition of other innovative components to enhance the technology, an analytical characterization system may be derived to provide extreme sensitivity and specificity. The purpose of this proposal is to develop fully the feasibility elements demonstrated in Phase I, leading to the development of a field portable method for the characterization of pathogens and their indicators in water.

Coliform bacteria may be used to monitor the bacteriological purity, and attendant potability, of water supplies. Coliform bacteria have been shown to be a marker of potential human fecal contamination and therefore of the possible presence of related pathogens (9-11). The coliform organisms are the principal indicator employed to assess the quality of drinking water relative to microbiological species (12). Among coliforms, fecal coliforms and *Escherichia coli* are all used to indicate fecal pollution, but *E. coli* is sometimes preferred as an indicator of recent fecal contamination and of the possibility of enteric pathogens, since they often coexist with fecal coliforms or *E. coli*. Remaining members of the coliform group - *Enterobacter*, *Citrobacter* and *Klebsiella* - are isolatable from feces, but their presence is not always indicative of fecal contamination. Previous methods of coliform determination have suffered from the long-term

need to maintain reliable supplies of 'standard' bacteria populations, and from the use of culture media and potentially useful but non-culturable bacteria that can require several days to assess (4). Other recent approaches to the determination of coliform bacteria in aqueous systems include those processes based on the activity of beta-D-glucuronidase activity (13-15). However, these latter systems have not been conclusively shown to provide highly efficient and specific determinations at requisite levels of sensitivity, and in some cases fail to detect a significant portion of the *E. coli* because of high occurrences of beta-D-glucuronidase-negative *E. coli* (16).

In January 1991, the EPA approved two tests for the detection of E. Coli that are positive for coliform bacteria (3). Subsequent to this approval, two studies were published that compared commercially available defined-substrate coliform tests for the detection and recovery of E. coli in water (12,17-19). Among the tests, the authors found that all methods were capable of detecting l cfu/l00 mL of E. coli, and no significant differences in detection were observed among the methods.

More recently, evidence has begun to appear that indicates gene amplification techniques may provide a partial (and critical) element to solve the problem of efficient monitoring of *E. coli* and related pathogens in water systems. Coliform bacteria have been detected in water by polymerase chain reaction and gene probes (20), *Legionella pneumophila* has been detected in water using a system based on amplification of a chromosomal DNA sequence (21), the rbcL gene from dissolved and particulate DNA has been amplified from algal isolates in aquatic environments (22) and a method has been developed for monitoring genetically engineered microorganisms in the environment (23). Numerous methods and related 'DNA amplifier' equipment have been described in the literature to provide the requisite temperature cycling (24-27), and equipment is

available commercially.

Most recently, during the last two years, studies of coliforms, viruses and other pathogens in water have been published that utilize PCR amplification technology. These include the determination of *Campylobacter jejuni* and *Campylobacter coli* in water (28), and *E. coli* in potable water (28). The first of these two PCR investigations employed conventional gel separation and photographic detection, and was able to demonstrate a theoretical sensitivity of 2 campylobacter cells per ml, using a sample of 100 ml. In the second investigation, a nested PCR protocol was used to enhance sensitivity, and was combined with ethidium bromide visualization of the PCR products for a sensitivity of 1-10 bacterial cells /50 ml water sample, in 6-8 hours. The nested PCR protocol was claimed to provide greater sensitivity by using the product of the first PCR as the template for a second round of PCR, which thence produces a fragment internal to the first set of oligonucleotide primers. However, none of the studies thus far appears to have considered a systematic, logical, analytically holistic approach to the minimization of time required in each step of the analysis process, while maximizing sensitivity. Nor is any of these procedures amenable to portability.

An interesting possibility for potentially improving the versatility of the PCR detection process has been illustrated very recently by the use of a multiplexing approach to the monitoring of multiple pathogens in water (30). In the multiplex approach, internal fragments of the respective genes, and the identity of the PCR products was confirmed by restriction endonuclease digestion. Using this approach, toxigenic and non-toxigenic strains of *V. Cholerae*, *S. Typhimurium* and *E. coli* in artificially spiked seawater samples were co-detected with a turnaround time of five hours. Conventional gel electrophoresis and photographic detection were used, and an estimated

sensitivity of about 1,000 cells was observed.

The advantage of gene probe amplification is that a species-specific nucleic acid sequence can be replicated (amplified) many times during a period that is short relative to the approaches described earlier that utilize culturing and other time-consuming techniques. The technology requirements for gene amplification are relatively simple compared to other methods of characterizing biomolecular activity, such as mass spectrometry or other related spectroscopic methods. To utilize the gene amplification technique, it is necessary to isolate the DNA from the candidate species (*E. coli* in this proposal), amplify a specific segment of the DNA using primers and a temperature cycling mechanism, label the amplified material with a tag appropriate for detection, and finally separate and quantify the tagged nucleic acid segment.

In this Phase II SBIR project we have developed further the components of a gene amplification-based capability, whose basic feasibility components were demonstrated in Phase I, leading to the development of a field portable analytical system for the characterization of *E. coli* in water.

BODY

The technical portion of the report in this section is organized around the technical objectives as they were originally proposed in the Phase II SBIR proposal. Subsequent deviations from the original research plan are detailed in each of the objectives. An overall goal of the objectives was to produce a PCR-based analytical process in which the total time required for an *E. coli* determination in water was less than two hours. The procedure actually developed in this project permits a complete determination within three hours, employing processes and instrumentation that are easily amenable to inclusion in a field-portable unit. Suggestions for expediting this process further are discussed in the CONCLUSIONS section of this report.

1. Investigate and improve the selectivity to clean up real water samples prior to *E. coli* detection.
    a. Assay a broad variety of water samples, including the EPA complex samples

This objective was modified in part to accommodate suggestions that arose from meetings held during the project among project personnel and Army technical and staff representatives. It was felt that the scope of the project and development of the technology were perhaps premature to permit a fully characterized survey of water samples, which would include the need for a more complete validation of the technology. A project that would permit a broad survey of coliform bacteria in water was suggested as a follow-on project to the current Phase II SBIR project.

In lieu of a broader survey, a decision was made to obtain water from a nearby mobile reverse osmosis water purification unit (ROWPU) that was located in Ft. Belvoir, Virginia. Since the

Army has a large number of reverse osmosis purification units in operation, and this would potentially be a major application area of the technology, the water samples from this unit were obtained toward the end of the project.

The research and demonstration ROWPU was located near the Potomac River. Four separate water samples were obtained: 1) Feed water sample. This sample was obtained by throwing a tethered plastic bucket from shore into shallow water in the adjacent Potomac River inlet and pouring a portion of the sample into a 250 ml disposable sterile Erlenmeyer flask and sealed. Identical sterile containers were used for all water samples; 2) Brine sample . This sample was obtained from a brine output mounted on the ROWPU unit, in an identical container; 3) Output water sample. This sample was obtained as the output of the total ROWPU purification process; 4) A membrane scraping from one of the reverse osmosis cylinders. To obtain this sample, a cylinder was removed from its holder in the ROWPU, and a circular saw was used to remove a pie-shaped segment of the rolled cylinder, also stored in a sterile flask. Several membrane layers were pulled away, and a scraping was removed for determination of *E. coli* bacteria.

b. Optimize the filter and prefilter system for separation of the *E. coli*.

The most important part of this process is to permit the isolation of the bacteria from the water sample and from other DNA-containing fragments. The typical size of the *E. coli* cells (a few microns) lend themselves easily to separation using a membrane filter of sufficient porosity to permit efficient water transport, but with small enough pores to retain the bacteria.

An additional screening process was investigated, partly as a result of discussion with Army personnel, as a means of screening the presence/absence of DNA- or RNA-containing material in water systems. Using this approach, the complete absence of genetic material could be

interpreted as a means to verify the purity of water, based on the absence of any pathogenic species that would by definition contain genetic material. Although these DNAs may not be fully characterized by this assay, it can still be useful to detect a contamination. To accomplish this goal, the presence of DNA or RNA was screened using arbitrarily primed PCR (AP-PCR) to initially identify the presence or absence of genetic material. A primer with an arbitrarily selected sequence was used to produce a DNA "fingerprint". The AP-PCR technique was used to detect the presence or absence of bacterial and viral pathogens(31-33), and has also been used in this project to detect the presence of *E. coli* bacteria in spiked and collected water samples. Patterns generated by AP-PCR (31,32) have been used in identifying different strains of mice and genetic mapping. An example of patterns produced by several pathogens, including *E. Coli*, *H. Pylori* and *P. Falciparum*, as well as human sample patterns, is summarized in Figure 1.

If we use this screening process as the first step in a hierarchical process to screen for groups of species, a positive result would require a specific PCR analysis to identify the source(s) of the DNA. The species-specific analysis would be based on a gene with a known, unique DNA sequence for each pathogen. For example, the *H. pylori* urease A gene can be used, and the *fla A* gene can be used for the *campylobacter* species.

2. Implement microwave technology for the efficient lysis and PCR amplification of *E. coli* DNA.

   a. Improve the efficiency of the lysis process

      i. Optimize the microwave parameters to minimize the time required for lysis.

This objective component was not pursued in the detail originally proposed, since the PCR amplification process was judged not to be sufficiently effective for the amplification of DNA from bacteria. Earlier tests with DNA from human samples appeared to function quite effectively, but the transfer of microwave amplification to bacteria samples was not accomplished efficiently.

As originally envisioned, the microwave technology was intended to be a single source of energy used to perform multiple functions efficiently: lysis and PCR amplification. Usually the lysis process has been accomplished by boiling the sample in water for a period of time, after which the cell wall(s) may be sufficiently degraded that some of the DNA leaks out that may be used for characterization. As has been observed, the cell walls of bacteria and viruses (e.g., *Staphylococcus aureus*) can be relatively strong structures, and lysis by boiling is ineffective (30). Since the amplification process is an absolutely necessary part of the water analysis process, and we were not able to make the microwave function effectively for bacteria, we abandoned for this project the intended microwave lysis-amplification in favor of a more conventional energy source for the PCR amplification alone, using Peltiér thermoelectric devices (described elsewhere in this report).

The intended function of microwave for lysis was to use a non-aqueous, low dielectric constant, liquid support medium for the bacteria following isolation by filtration. In the low dielectric medium, each bacteria cell would become individual water (high dielectric constant)

containers that could selectively and efficiently couple with the microwave radiation, resulting in an explosive water vapor rupture of the cell wall. In the proposal we referred to this process as 'flash lysis,' an almost instantaneous process that would contribute significantly to the shortening of the overall water analysis time required. Although the microwave PCR amplification was abandoned for this project, it still may be desirable to use a miniature microwave radiation source as a separate power source to accelerate the lysis process. However, there may be other approaches to improve lysis efficiency that utilize the thermoelectric process as well. These are discussed in the Conclusions section.

ii. Investigate chemical modification and fluid medium techniques to enhance microwave absorption.

A standard CEM Corp. microwave oven (Model MDS-2000) was modified at the factory to our specifications that permitted the microwave power to be shifted discretely between or among two or more power levels (and therefore two or more temperatures for the PCR amplification). Further, the changes were incorporated in the oven in the form of externally controllable software/hardware driven by a menu selection on the front panel. With this reconfigured system, the oven could be operated as a 'thermal cycler' to repetitively and reproducibly cycle between or among temperatures. The temperature in the PCR amplification mix, or reference solution, was monitored with a shielded thermocouple that is normally supplied with the oven.

To simplify the PCR amplification process and thereby shorten the time required to obtain a given number of thermal cycles, the conventional three (step) temperature process was replaced by a two (step) temperature process. Using this approach, empirical tests were performed with

the microwave oven system adapted for PCR amplification. A two-temperature process was adapted for cycling between temperatures at 55° C and 90° C. A sample of the microwave temperature cycling steps, as monitored by the thermocouple in a standard 100 μl tube, is shown in Figure 2a. The temperature shifts are not as fast as those obtainable with commercial Peltiér device-based systems, but nothing had been done physically to redesign the system to promote heat transport during the temperature cycling.

Attempts to improve the microwave cycle time response (i.e., minimum temperature rise and fall times between cycles) were tried empirically by changing buffer formulations. Several solutions of varying proportions in 10X buffer formulations, including Tris, KCl, $MgCl_2$, and pHs, were compared with an Epicenter buffer formulation:

500 mM Tris (pH 9.0)

200 mM $(NH_4)_2SO_4$

15 mM $MgCl_2$

The Epicenter buffer formulation worked best, and the microwave-based thermal cycling plot using this buffer is shown in Figure 2a. The relatively sharp temperature rise and fall times compared to other buffer combinations or to water, may be due to a higher ionic strength solution that couples more strongly with the microwave radiation. A less efficient thermal cycling plot typical of the other formulations is shown in Figure 2b. The remaining formulations exhibited response times that were similar to or worse than that shown in Figure 2b. As with any thermal cycling system, the ability to absorb and dissipate energy is important for rapid rise or fall times. Correspondingly, thin-walled PCR tubes were more efficient than thick-walled tubes.

This system and approach was used successfully for the PCR amplification of DNA, and all of

the early work with this system was accomplished using human DNA. The switch to bacterial DNA, with associated primers, was not successful in that the sensitivity of the process was limited. Repeated experiments with the microwave-based amplification process for bacteria, using different primers, were also not successful. The problem was not decipherable in a reasonable amount of time, and this approach was abandoned in favor of a more conventional amplification process.

In spite of the apparent overall lack of success with the microwave approach, there were other small successes. One significant accomplishment was the successful lysing of the bacterial cells by microwaving directly in the PCR mix. This eliminates the need for a separate lysis step. The dilution of a known number of bacteria from $1x10^9$ cells/ml to 0 cells/ml, resulted in the ability to detect, by ethidium bromide staining, between 10^4 and $5x10^5$ cells/50 μl PCR mix. Higher cell concentrations were not detectable, and the presumption was that the cell debris was interfering with the PCR mix beyond the higher cell concentration level. Regardless of these effects, it is believed that the direct lysis/amplification by microwave could be a very simple and useful process to screen bacteria containing plasmid without the need of doing "mini-preps".

iii. Review and determine available microwave sources to meet the requirements of 2.a.i.

Since the microwave approach was eventually abandoned for amplification, only a cursory survey of microwave sources and designs was completed to identify manufacturers and service providers. The actual design and implementation of a specific miniaturized microwave source for the field prototype was scheduled to be deferred until Phase III, since it was beyond the scope of the Phase II project.

3. Improve detection sensitivity and develop a simplified detector system.

a. Modification of the protocol for amplification of *E. coli* lack and lamb genes.

Following the work of Phase I, a three-step PCR was adapted for the lacZ gene, utilizing B.L. Taq polymerase and a simplified PCR buffer. The magnesium concentration was optimized. Following the confirmation of earlier work, a two-step PCR process was developed using a PCR machine for eventual adaptation to the microwave conditions. This two-step program was developed for two reasons: 1) To establish two "target temperatures" (as opposed to the Phase I three temperature PCR program) for the microwave oven to accomplish and 2) to reduce the temperature difference between the two "target temperatures." The following program has been used successfully for the amplification of the bacterial lacZ gene:

PCR Conditions:	PCR Program:
20 mM Tris-HCl, pH 8.4	90° C for 1 min, 30 sec
50 mM KCl	55°C for 1 min
0.32 mM each dNTP	35 cycles
2.5 mM $MgCl_2$	
40 pmol primer ZL1675	
40 pmol primer ZR2548	
250 ng *E. coli* DNA	

In the early portion of the project, the above conditions were used with radioactive labeling and photographic detection to detect the DNA from one *E. coli* cell.

Denaturation of bacterial DNA was accomplished in the microwave system by microwaving for 3 min at 70% power. This key experiment demonstrated that microwaving of Taq polymerase and other PCR amplification components appeared to have no effect upon subsequent amplification.

In anticipation of simplifying the amount of technical skill required of the ultimate user of the

'kit', several "premade" PCR mixes were made and assayed. A premade mix is the precursor to inclusion in a kit, and would allow the amplification reaction by permitting the user to add only the potentially contaminated water sample and the Taq polymerase enzyme required for amplification. The storage lifetime of such kits will be evaluated.

b. Develop a simplified detector/comparison system for semi-quantitative comparison of water sample qualities.

Amplified specific genes by PCR are generally analyzed by running the samples on agarose gel, stained with ethidium bromide and examined for the specific DNA under UV light. However, this procedure doesn't provide a specific diagnosis and is not applicable to field conditions. Hence a detection system based on enzyme linked immunosorbent assay (ELISA) has been developed which is faster,simpler and more sensitive than the conventional methods. This combines the specificity of the DNA amplification system by PCR with the sensitivity of enzyme-based detection system of the ELISA. This procedure has been used successfully for the detection of *Salmonella typhi* (34). The schematic diagram of the ELISA process is illustrated in Figure 3, using campylobacter as an example.

A pair of 5'-biotinylated primers and digoxigenin-labeled dCTP are used in the PCR reaction mixture. This allows the incorporation of biotin and digoxigenin groups into the amplified DNA during the PCR. As a result, the final product can be specifically immobilized to a streptavidin coated 96-well micro titer plates by biotin-streptavidin interaction and quantitatively assayed by a enzyme-linked colorimetric assay using a commercially available antidigoxigenin conjugate. This procedure is well established and easily amenable to high-throughput screening.

The oligonucleotides used for amplification of lacZ gene are synthesized with 5'biotin modification and used for amplification of lacZ gene during the PCR along with digoxigenin-labelled dCTP. PCR products are diluted in Tris buffered saline (TBS, pH 8.0) and transferred to a streptavidin coated ELISA plate and incubated at 37° C for 30 minutes to allow the binding of the PCR products. The plate is washed three times with TBS and incubated with diluted antidigoxigenin Fab/alkaline phosphatase conjugate at 37° C for 30 minutes. Following three washings with TABS, 100 μl of disodium p-nitrophenyl phosphate (1 mg/ml) in 10% diethanolamine (pH 9.8) is added. Absorbance at A_{405} was taken and used to estimate the number of bacteria in the sample by comparing with a standard curve drawn using absorbance readings taken from a known number of bacteria.

4. Assemble and test prototype analysis system

a. Combine the developments in objectives 1-3 into a prototype water analysis system

Having experienced difficulty with the use of microwave radiation during the PCR amplification portion of the project, we changed direction of the project with respect to a means to provide field-portable PCR amplification. The design selected for the required thermal cycling was based on the Peltiér thermoelectric effect.

The design of this instrument was intended to be adaptable to the rigors of field use as well as for use in the laboratory. Of necessity the instrument must be capable of convenient operation from mobile power sources; i.e., car batteries, and be light weight as well. Solid state devices fulfill these requirements nicely. The operating principle makes use of Peltiér effect cooling as well as thermoelectric heating to thermally cycle between or among the temperature control

points necessary to produce the conditions necessary for biological DNA acceleration to occur. An embedded computer/controller provides all operating protocols, thermal settings, precision timing, etc. A single push-button calls the desired protocol so that only limited access is given to the operator and requires no special skills or decisions except for loading the samples and initiating the process.

Electronic design considerations, especially the need to run from automotive power sources, suggests a twenty-four volt design. The power voltage level is adequate to operate the National Semiconductor LM12 power operational amplifier in a half bridge driver configuration. The six power IC's are mounted on their own heat sink with all the control circuitry located on a separate PC Board. Pairs of drivers power Peltier modules with sets of two devices coupled in series, having a total of six devices arranged in close contact on the heat sump. The sample holder is situated atop the arrangement with all surfaces being coated with thermally conductive grease. The control electronics resides on the PC Board. A Texas Instrument TSS400 computer/controller chip along with associated memory chips provide the process control and timing. An LCD display provides continuing monitoring of operating status and a four key push-button pad provide operator input. Output from the control drives an eight bit DAC which sources the voltage set points for the analog control loop. Temperature measurement is done with thermocouples located on the sample holders and connected to an ADC located within the TSS400 controller. A separate thermal circuit provides an electronic "ice point" for reference.

In operation the samples are held at temperatures ranging from 50 to 95° C., alternating from high to low in short time periods and back again. The object is to switch between the temperatures as quickly as possible so as to reduce the overall time required for a series of cycles.

The outcome of tests is shown in the accompanying graphs shown in Figure 4, that illustrate the rise time and fall time. The two traces represent the temperature of the block (heat sump), with attendant sharp rise and fall times, and the temperature of the actual solution in the PCR cell as monitored by a thermocouple. This lag time is typical of this type of thermoelectric device, although we believe it may be improved significantly by dramatically redesigning the configuration of the PCR cells.

The construction required a large heat sump or reservoir with the Peltier devices tightly thermally coupled providing a ready source of sink of heat. To this end a block of aluminum approximately 4 inches by 7 inches by one inch thick is mounted in the center top of the cabinet. The solid state devices are attached by a spring holder to allow for uneven thermal expansion. Individual sample holders are attached to a copper plate spanning the array of modules to even out any thermal differences. This arrangement allows for alternate styles of sample holders to be used.

Photographs of the completed thermal cycler system are shown in Figure 5. The unit is easily portable, and may be further simplified and made more lightweight, depending on the ruggedness requirements of the Army portability specifications. Since the electronic design was accomplished in-house, the thermal cycler may also be integrated with other potentially electronic-powered components of a final prototype portable analysis system. The thermal cycler shown in Figure 5 a,b has been used extensively in the laboratory for PCR amplification of the *E. coli* samples for this project since its production, and has performed equivalently for the required purpose compared to more expensive commercially available systems. The results of the performance of this simple unit for PCR amplification of the lacZ gene are compared with a more expensive

commercially available unit and shown in Figure 6. The circuit diagram for the unit is shown in Figure 7. A low (price) end version spawned by the simple design of this unit has been prototyped and is being set up for commercial production.

An important consideration for the design and construction of this unit (Figure 4) in house - instead of modifying an existing commercial system to perform the tasks for this project - is that it provides the technical basis for modifying the design for inclusion in a field prototype water analysis system that is specifically dedicated to the Army water characterization objectives. Working at the electronics design and fabrication level also is expected to expedite the integration of other electronic and power supply components of the water analysis prototype as the measurement requirements change.

b. Evaluate the operating parameters of the system using the range of water samples employed in objective 1.

The colorimetric PCR-ELISA process was calibrated by producing a dilution series of E. coli cells, ranging from 10^7 to 10^0 cells. The result of the calibration is illustrated visually in the photograph of Figure 8, in which all concentrations of cells are easily visible, including the container with 1 bacterium. These results are contrasted in Figure 8 with positions A5 and A6, which are negative controls and show no evidence of the presence of bacteria (i.e., they are clear).

Using this PCR-ELISA process the ROWPU samples discussed in objective 1 were analyzed for the presence of *E. coli* bacteria. Each of the samples: Feed, Product, Brine and Membrane, were filtered through 0.2 μM nitrocellulose membrane (Nalgene membranes) and both membrane and filtrate were analyzed with PCR. Membranes were boiled with 5 ml of distilled water to lyse

the DNA and 10 μl of this material was taken for gene amplification. Ten μl of filtrate was also used for analysis after boiling for 10 minutes. PCR samples were analyzed by ELISA and samples were graded according to the color developed after comparing with color from positive controls with known numbers of bacteria as follows:

	filtrate	from filter
1. Feed	-	-
2. Product	-	-
3. Brine	-	+++
4. Membrane	-	-

10^5 bacteria +++	10^2 bacteria +++	10 bacteria ++	1 bacterium +

It is not clear from this brief study whether the bacteria found in the brine is an accumulation of material, perhaps a residue from larger quantities of processed water, compared to the sample of Feed (Potomac River) water. One might have expected to find bacteria contamination in the Feed water. However, it is possible that the bacteria concentration in the Feed water was not high enough to be detected. The results here are interesting, but more work will be required to study the purification process systematically.

CONCLUSIONS

The completion of this project has resulted in the development of PCR-based procedures and instrumentation that collectively permit the detection of *E. coli* at concentrations and sensitivities sufficient for the EPA requirement of one cell per 100 ml of water. We have observed that gene amplification when followed by conventional DNA detection system could detect up to 10 bacteria. However, when PCR is combined with ELISA for detection of PCR products, the sensitivity level increased to one bacterium. This system is simple, doesn't require higher technical skills and can be easily adapted to field conditions. Moreover, this detection system is amenable to automation and thus very useful for large scale screening of water samples. Further, the individual technology components are eminently suitable for collective inclusion in a portable system to be used in military and other field operations. We have projected a combination of the technical components in a portable unit as depicted in Figure 9. Based on the weights and sizes of the several components, we believe that an assembly can be completed within the original RABTEK specifications of 26 pounds, if this is the required configuration. Alternately, it may be assembled as part of a portable test kit for the private sector.

The tasks that remain to be completed prior to the actual production of a usable field prototype are believed to be 'second-order' tasks, none of which is expected to scientifically or technically preclude full development of a viable system. However, we do recognize that a substantial program of highly focused and aggressively executed work remains to be done to realize the goal of a field-usable system in the very near future. The essence of the original insight of a few years ago that led to the RFP for this project has been realized here. In parallel, other

research laboratories have begun to recognize the potential of the technology, and have published studies, albeit fragmentary with respect to overall time and ease of use considerations. These other studies (5-8, 20-29) have supported the principles involved in the execution of this project.

Beyond the current work, there are many improvements that may be effected and questions that need to be answered. One way to organize the discussion of these points is to begin with the sampling step and progress through the total characterization process:

SAMPLING

The eventual success of any analytical process used to measure real samples may be limited initially by the sampling step. In trace analysis, care must be taken to avoid contamination from extraneous materials. This may be accomplished by using only clean (sterile) containers, reagents and tools. A sample of pond water is likely to contain any number of organisms unrelated to the pathogenic organisms. One way to deal with this problem is to screen first for the presence of DNA/RNA-containing species (31-33), followed by specific protocol(s) to define the presence of specific pathogen(s). A judicious selection of filter arrangements in the initial filtering of the sample may preclude some of these problems. If the predominant Army application is to monitor the purity of ROWPU water, then the filtering requirements prior to lysis, PCR amplification and detection would be simplified.

An additional question that has been raised is the ability to distinguish between viable organisms (i.e., E. coli cells) compared to DNA fragments that contain the amplifiable portions. The primary answer to this concern can be found in the use of filters to size-screen the bacteria (nominal microns dimensions) from the much smaller (non-viable) DNA fragments that pass

through the filter with the rest of the water sample.

LYSIS

Most of the lysis procedures used in the PCR-based coliform determinations reported in the literature utilize boiling of the sample in water, with or without additives, or the use of freeze-thaw cycles to enhance the efficiency. As the overall analysis time required for the coliform bacteria decreases below two or three hours, the several minutes typically required for lysis using conventional approaches becomes a significant part of the overall time required. For the analysis of *E. coli*, the cell wall is relatively easy to lyse in a few minutes. Other pathogens that may be added to the list by Army representatives have been shown to have stronger cell walls that are more difficult to lyse(30).

In addition to the implementation of the microwave 'flash lysis' using dielectric constant differentials, we have also discussed the possibility of using ultrasound to assist in the lysing process. Using a separate Peltiér device for the lysing chamber, the sample containing the cells would be cooled to freeze the cells, and ultrasound would be used to rupture the cell wall quickly, followed by return ot the solution temperature to ambient.

PCR AMPLIFICATION

The primers used for amplification in the E. coli detection process have not changed since the earlier work of the project. Unless there is a compelling reason to change to different primers and a different amplification process, the existing choices appear to be adequate for the determination of coliform bacteria. If a decision is made later to change to additional pathogen monitors, then it

would be appropriate to rethink the approach to the selection of primers.

DETECTION

Initially the work in this project was accomplished using gel separation and photographic detection as a means to demonstrate the functionality of the procedures for lysis and PCR amplification and detection sensitivity. The development of a simplified detection process that did not require photographic detection and gel separation was essential to the portability of the technology. The colorimetric detection procedure described in the report is suitable now for the detection of coliform bacteria using protocols that can be adapted to field use. No doubt there will be subsequent improvements in both the speed and sensitivity of the colorimetric processes employed for detection, and these may be added as they evolve.

The addition of 'dip-stick' technology is conceptually simple, but in practice may be more difficult because of the relative complexity of handling the number of individually coated papers employed for detection over a range of concentrations.

VALIDATION

The technology employed here, and the subsequent improvements and modifications, represent potentially a quantum level advance in the ability to characterize pathogens in water. However, the technology is too new and untested to have been accepted as a standard measurement procedure by the EPA. Therefore, to be employed in the Army (and other DOD component) water programs, the technology must be validated for the ability to reliably characterize water samples. This is a task that could be taken on by the Army to expedite the movement of the

technology into the field as soon as possible. Validation within the Army also would expedite the acceptance of the technology within the EPA as a standard, or standards, and would also accelerate the expansion of the new technology into the private sector as well, for which a substantial commercial market exists.

Commercialization Activities and Plans

We believe the technology developed in this project has very significant commercial potential. In parallel with the completion of this project, we have initiated discussions with investors in the private sector. Among these is the Maryland Health Care Product Development Corporation (MHCPDC). MHCPDC is the operating entity of a public/private partnership funded by the Department of Defense (DOD) Technology Reinvestment Program and the State of Maryland. This corporation invests (with matching private sector funds) in health care technology developed within the DOD.

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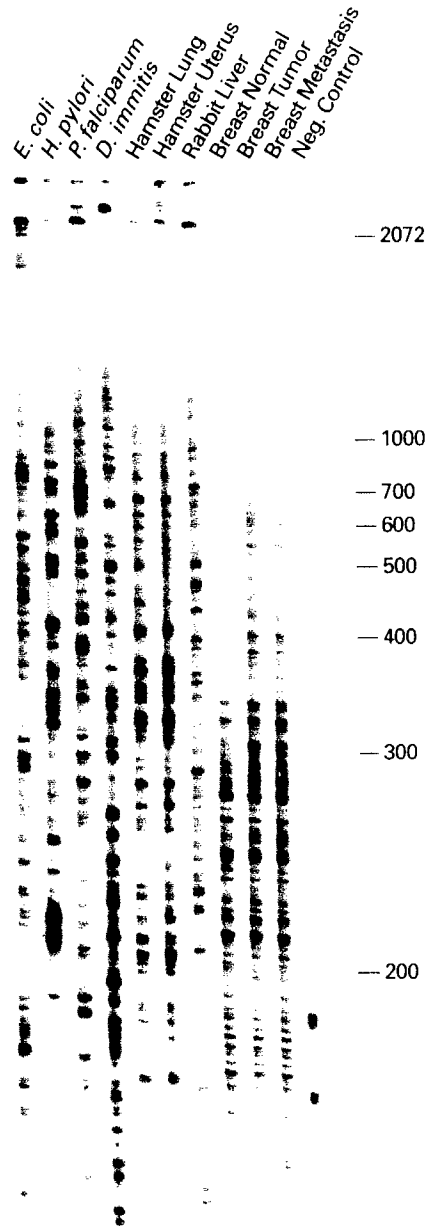
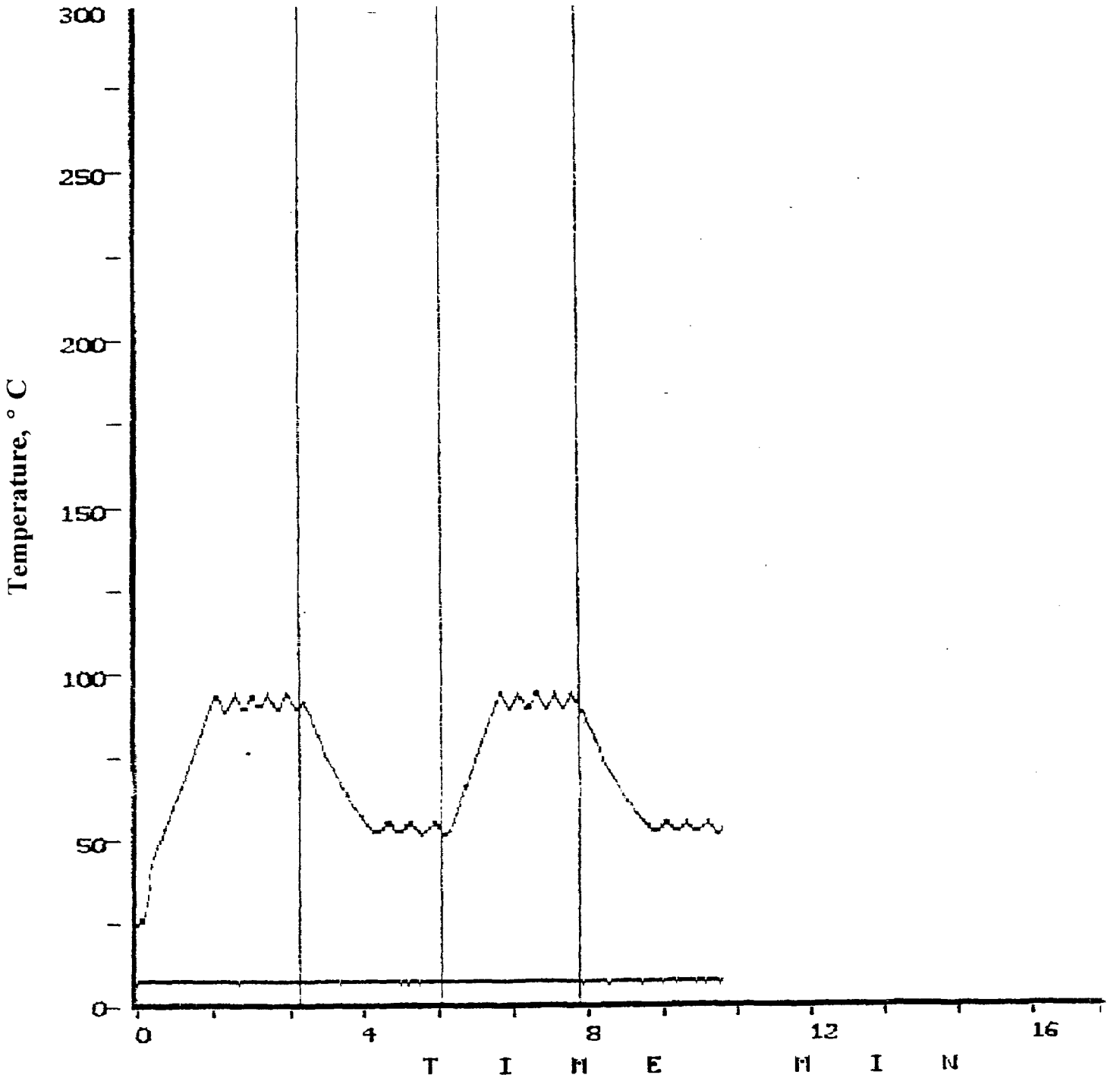
List of Participants

Dr. Robert Bohn
Dr. Terri Lehman
Mr.Rama Modali
Dr. Larry Moore
Dr. Muralidhara Padigaru
Mr. Hanxiang Si
Ms. Zhong Wu



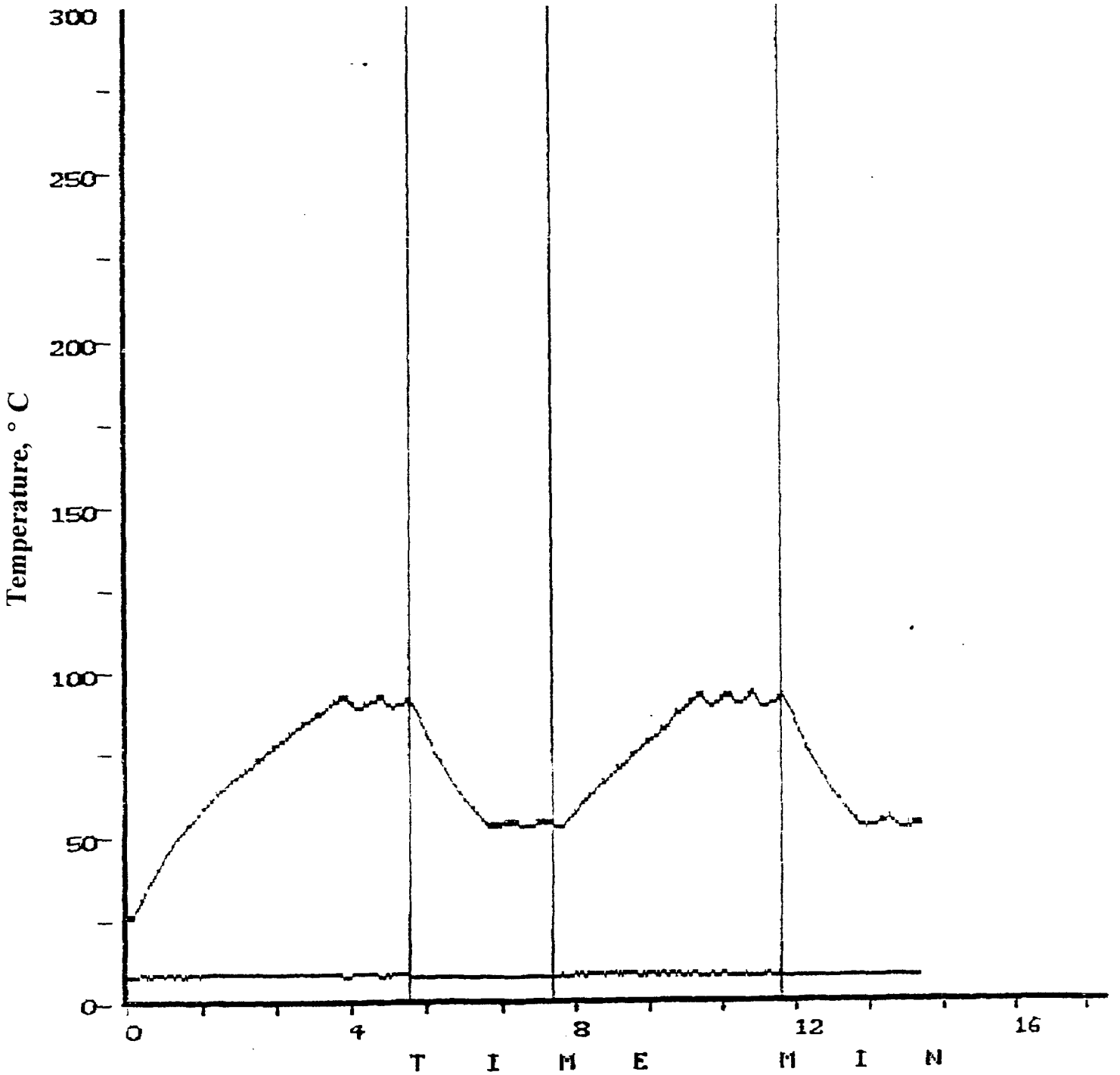
Amplified with Stoffel Fragment Using End Labelled Primers

Figure 1: An example of separation patterns produced by several pathogens and human samples.



Microwave heating profiles of PCR buffer solutions using differing proportions of components.

Figure 2a: The optimum combination based on an Epicenter buffer



Microwave heating profiles of PCR buffer solutions using differing proportions of components.

Figure 2b: A typical heating pattern for a non-optimum buffer mix.

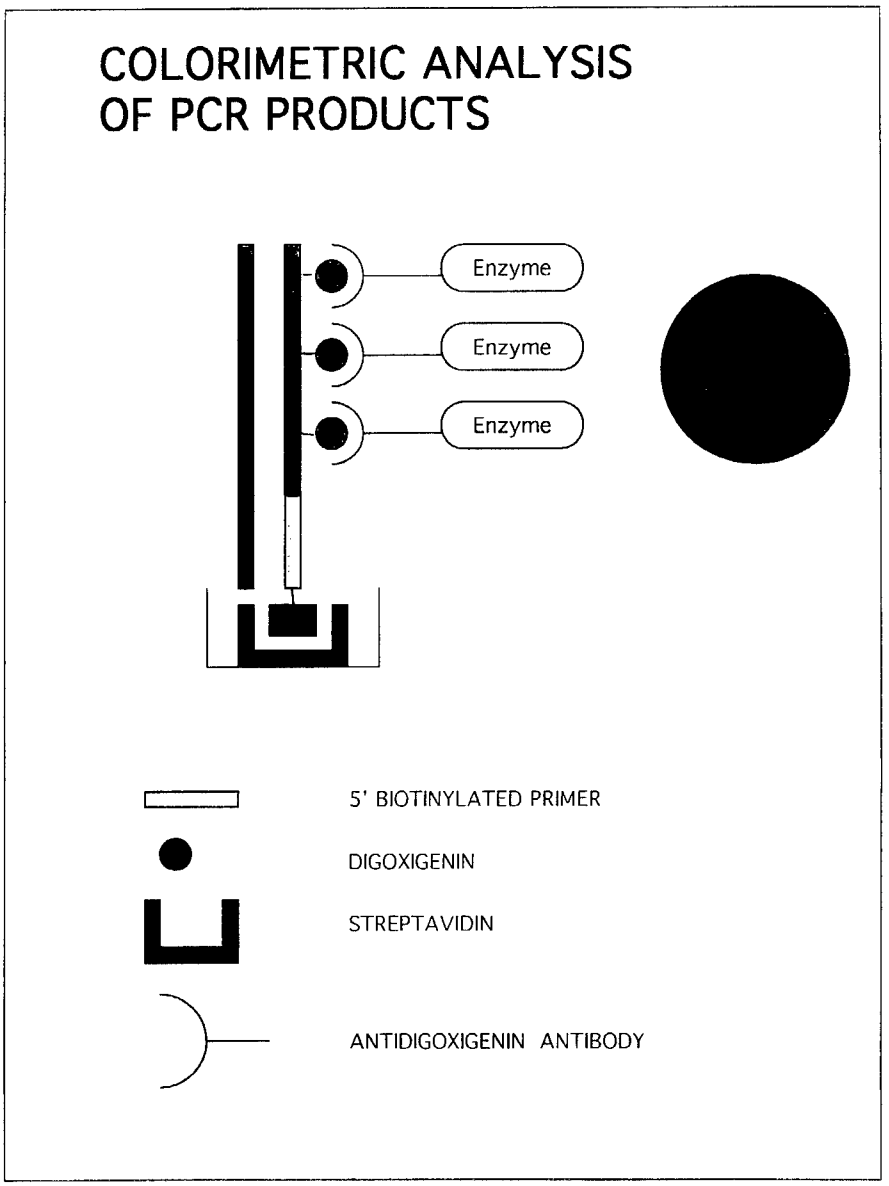


Figure 3: A schematic diagram of the PCR-ELISA technique for colorimetric detection, using campylobacter as an example.

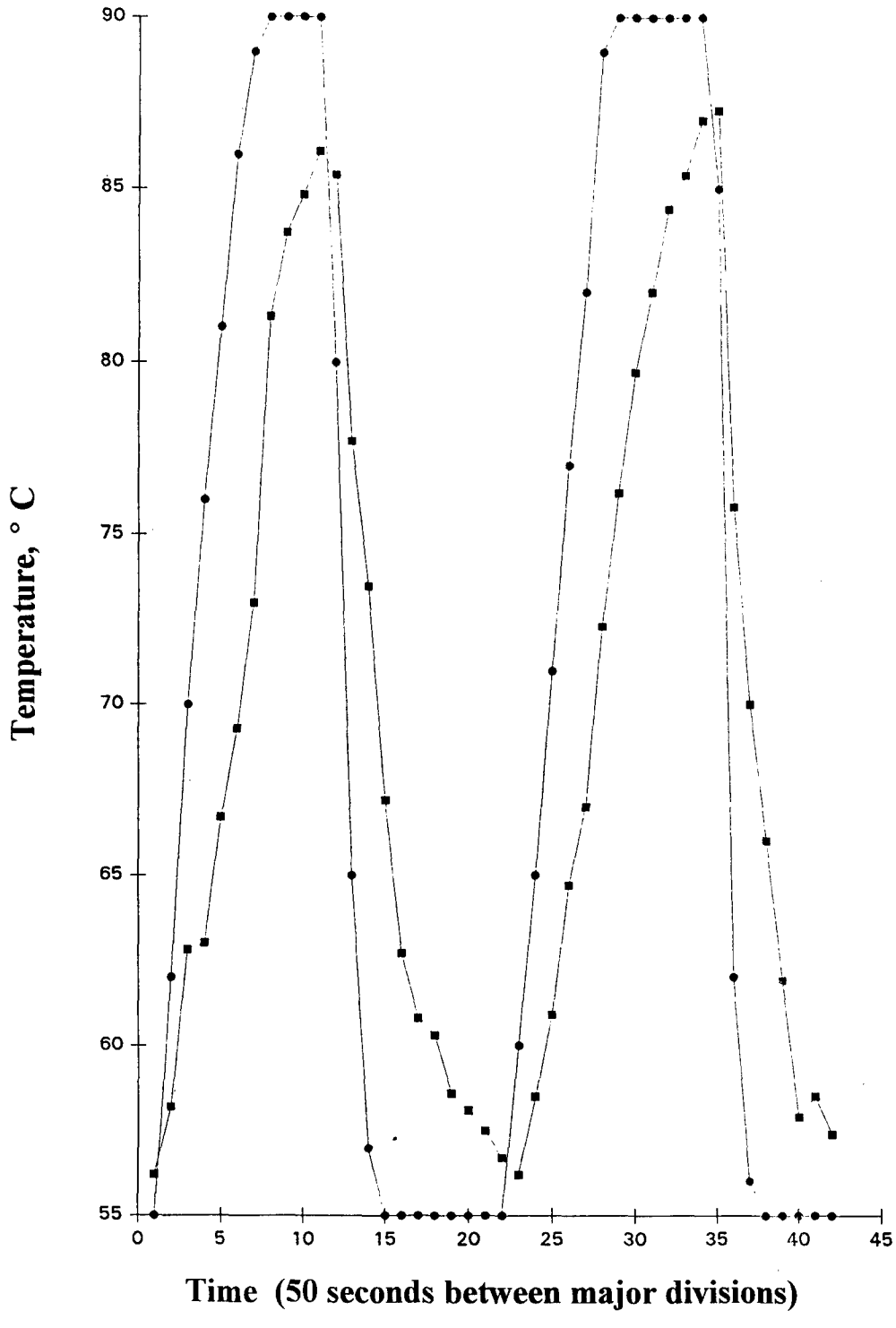


Figure 4: Typical heating patterns for the Peltiér-based prototype designed and built for this project. The more sharply defined rise and fall times are for the (heat sump) block; the slower heating patterns are for the temperature of the PCR solution.

Figure 5a: Thermal cycling unit for PCR amplification of water pathogens

Figure 5b: Disassembled view showing power supply (below); and microprocessor/controller and sample holder (above)

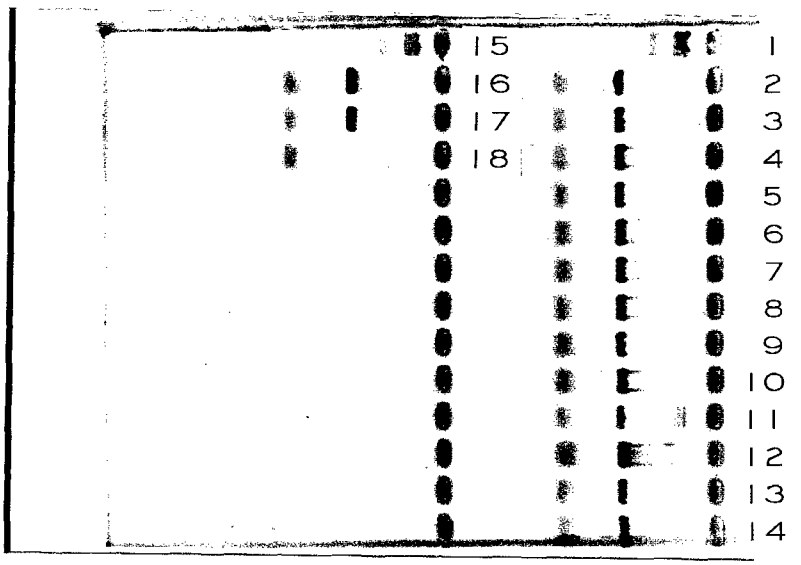


Figure 6: A comparison PCR amplification efficiency of the prototype thermal cycler built for this project compared to a commonly used commercially available unit.
A 1% agarose gel, using PCR samples for the lacZ gene was employed. Lanes 2 through 14 were produced with the prototype; lines 16 and 17 were produced with a commercially available unit. Lane 18 was a negative control.

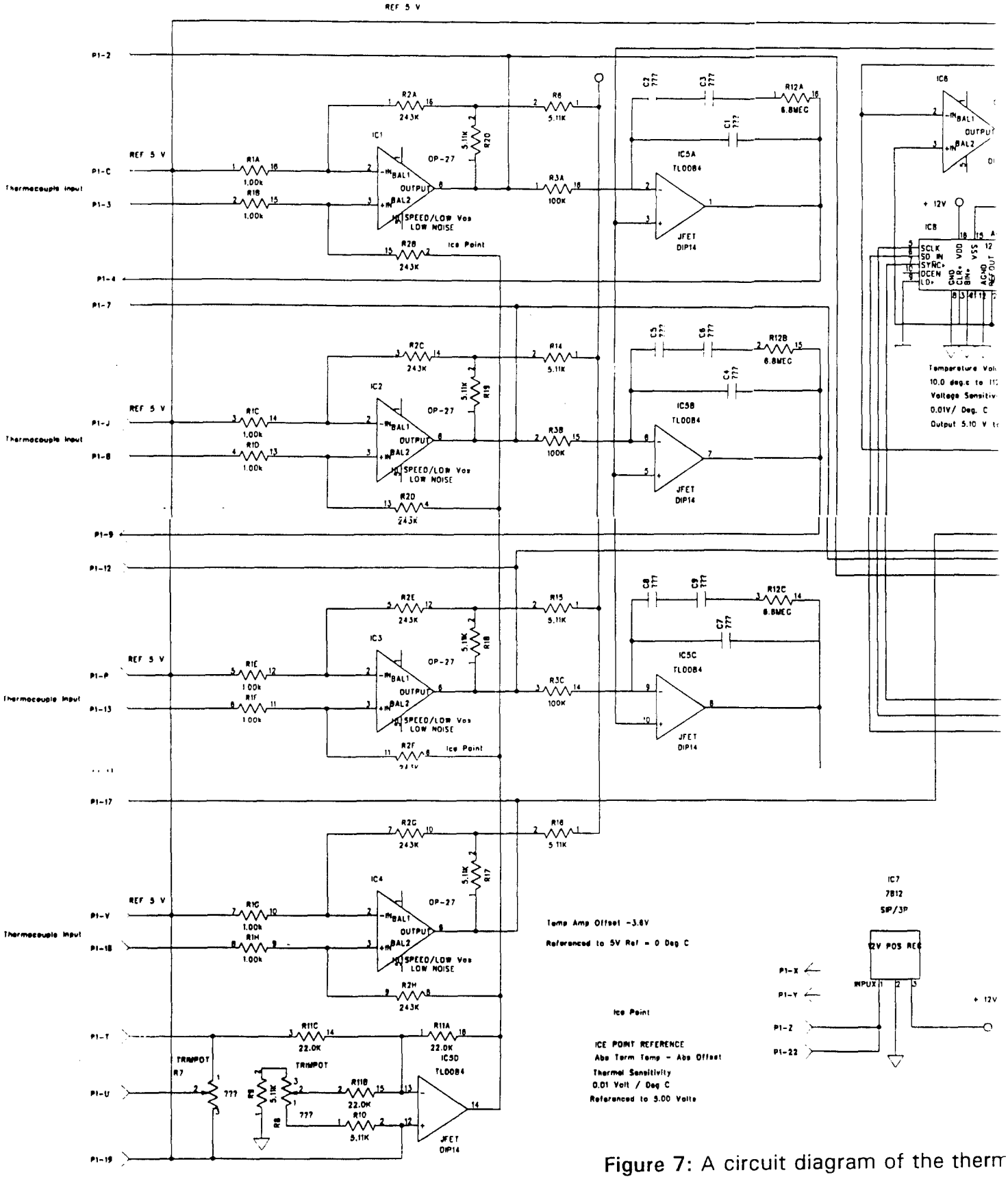
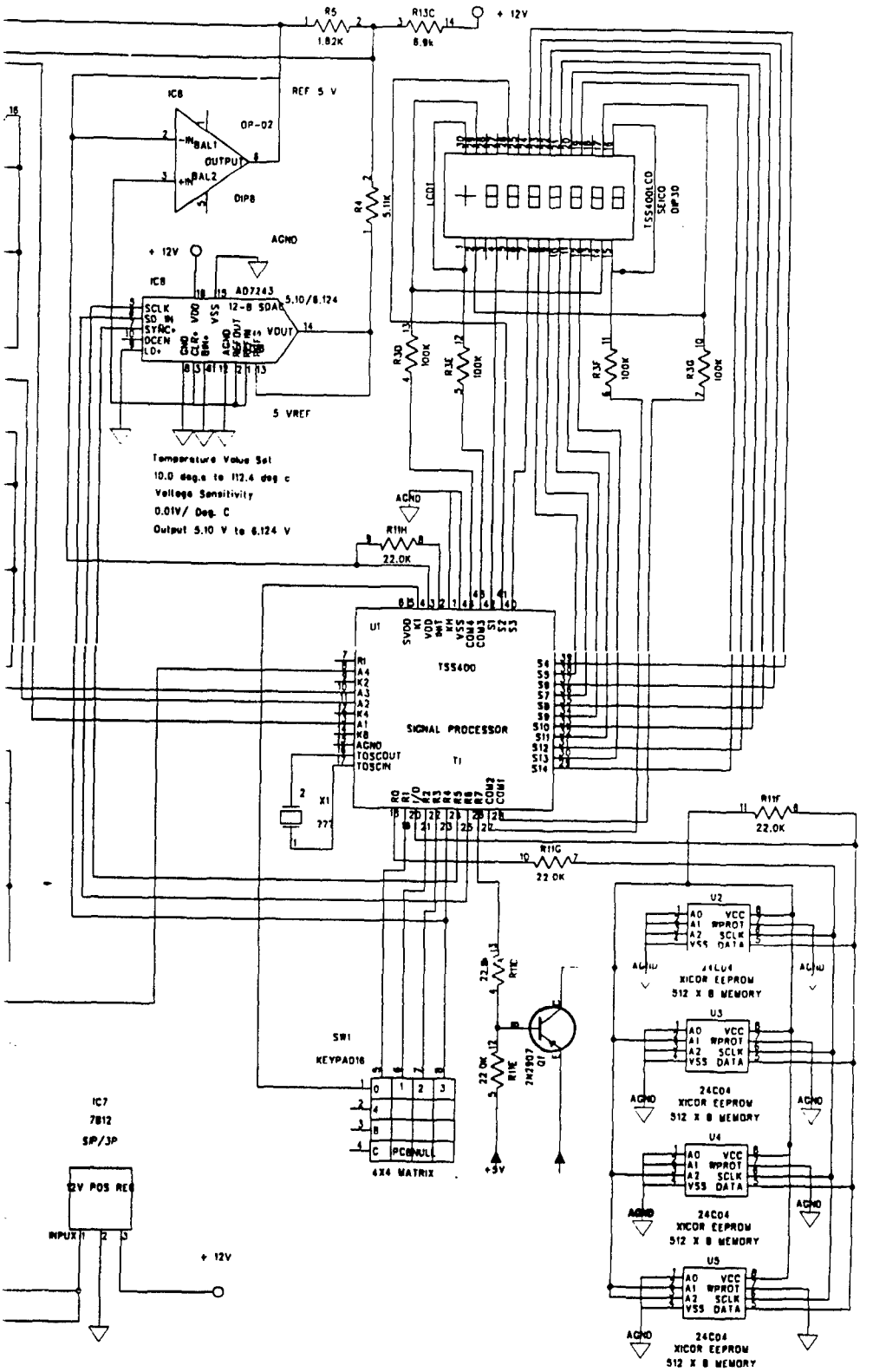


Figure 7: A circuit diagram of the therm



igram of the thermal cycler prototype developed for this project.

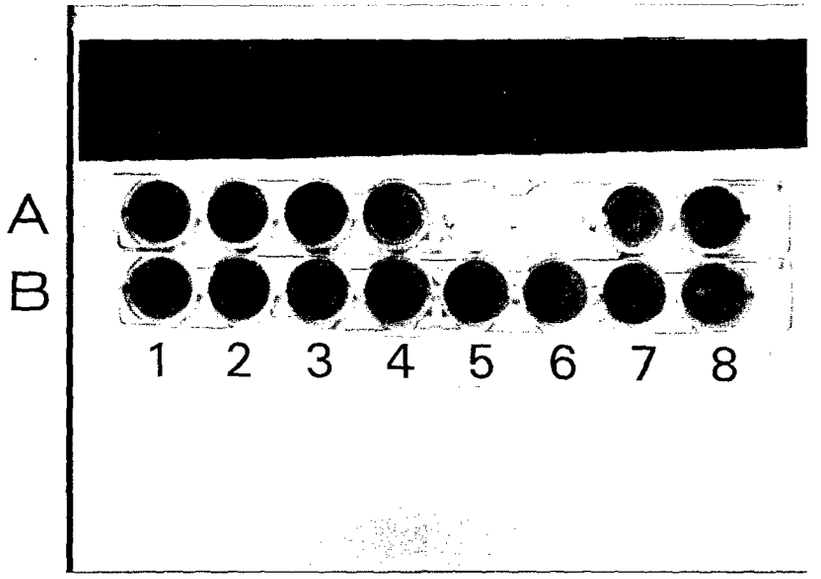


Figure 8: A calibration series to demonstrate the PCR-ELISA process for *E. coli* bacteria. Cells were taken at various concentrations, lysed, and used for amplification of the lacZ gene. Subsequently PCR samples were coated on microtiter plate and the color developed.
In row A: 1 -> 4, 10^5 -> 10^2 *E. coli* cells; A5 and A6 are negative controls.
In row B: 1 -> 8, 10^7 -> 1 *E.coli* cells.

Prototype Portable Water Analysis System

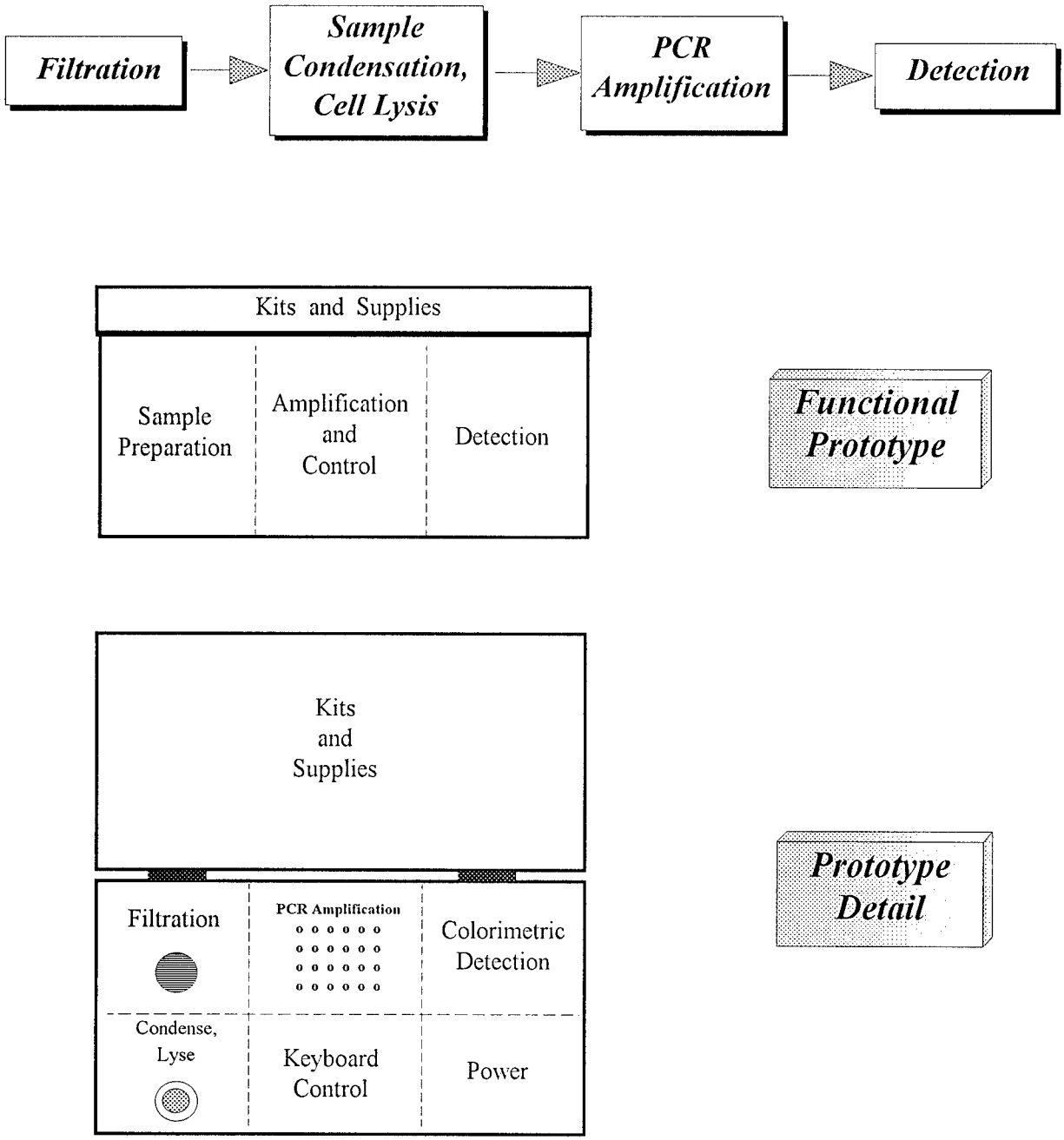


Figure 9: Schematic of a PCR-based water analysis process and prototype portable unit.

Rec'd 10/18/2000

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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

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